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Interaction of Ribosomal Protein S1 and Initiation Factor IF3 with the 3′ Major Domain and the Decoding Site of the 30S Subunit of *Escherichia coli*†

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ABSTRACT: We have studied the effect of the binding of ribosomal protein S1 and initiation factor IF3 on the accessibility of nucleotide residues 584–1506 in the small subunit of the *Escherichia coli* ribosome. Protein S1 strongly decreases RNase V1 attack at G1164, in hairpin 40 of the 3′ major domain, and weakly decreases DMS attack at C1302, in the central loop of the 3′ major domain, and at A1503, in the 3′ minor domain. It also weakly increases the DMS reactivity of A1004, in the 3′ major domain, and of A901, in the central domain. Factor IF3 strongly decreases RNase V1 attack (but not dimethyl sulfate attack) at A1408, in the decoding site, and weakly protects A1500, in the 3′ minor domain and near the colicin E3 cleavage site. Neomycin does not interfere with this effect of IF3, but IF3 interferes with the protective effect of neomycin against dimethyl sulfate attack at A1408.

Protein S1 is by far the largest of all ribosomal proteins. It appears to have only one site for RNA binding (Lipecky et al., 1977; Thomas et al., 1978; Yuan et al., 1979; Mulsch et al., 1981) and another site to interact with the 30S subunit or the all-protein phage-induced Qβ RNA replicase. The binding site of S1 on the 30S subunit is dependent on S9 (Laughrea & Moore, 1978b), more sensitive to trypsin than to RNase, insensitive to aurintricarboxylic acid (Boni et al., 1982; Odom et al., 1984), and salt-sensitive, unlike its interaction with RNA, which is very stable in 1 M salt (Carmichael, 1975; Draper & von Hippel, 1978b). Studies with fragments of S1 have shown that, by and large, residues 224–309 constitute the RNA binding domain of S1 while residues 1–193 constitute the ribosome and the Qβ replicase binding domain (Subramanian et al., 1981; Guerrier-Takada et al., 1983; Subramanian, 1985; Giorginis & Subramanian, 1980). However, residues 1–193 do not bind to 30S subunits as well as S1; they are required for its nucleic acid unwinding property, and they possess some salt-sensitive poly(U) binding activity (Thomas et al., 1979; Giorginis & Subramanian, 1980; Subramanian et al., 1981), not unlike S1's site I (Draper & Von Hippel, 1978a). Thus, S1 may possess a second RNA binding site with an association constant of less than 10^5 M^{-1}. Draper and Von Hippel (1978a,b) had earlier concluded the existence of two possibly noncontiguous RNA binding sites on S1.

In this context, it seemed justified to assess the impact of S1 on the chemical and enzymatic reactivity of a large number of nucleotide residues within the 16S RNA of the ribosome or the 30S subunit. We were stimulated by the knowledge that all other 30S ribosomal proteins (several of which not being known as RNA binders) have some impact on the reactivity of 16S RNA (Stern et al., 1988d). S1 is the only 30S protein left to be footprinted. However, our goal is different from that of Noller's laboratory: not so much to locate rRNA segments within the 30S subunit but rather to delineate the zone of influence of mammoth S1.

Though it is no longer believed that initiation factor IF3 plays a direct role in binding mRNA to the 30S subunit (Canonaco et al., 1989), IF3 has a number of attributes which justifies a simultaneous study of the interaction of IF3 and S1 with the 30S subunit. Both proteins unfold RNAs (Schleich et al., 1980; Bear et al., 1976; Thomas & Szer, 1982; Wickström et al., 1986), can be cross-linked to the 3′ end of 16S RNA without actually needing it for binding to the ribosome [see Laughrea et al. (1978a) and references cited therein], may induce small conformational changes in the 30S subunit (Michalski et al., 1978; Laughrea et al., 1978b; Beaudry et al., 1976; Pon et al., 1982; Gualerzi & Pon, 1990), and cooperate during the initiation of R17 RNA-directed coat protein synthesis (Steitz et al., 1977). IF3 seems to inspect the correctness of the interaction between the mRNA initiator codon and the anticodon stem–loop of the P-site-bound tRNA (Hartz et al., 1990; Gualerzi & Pon, 1981), and S1 differentially increases the affinity of the 30S subunit for various mRNAs (Steitz et al., 1977; Roberts & Rabinowitz, 1989; Boni et al., 1991). In contrast to S1, the binding site of IF3 on the 30S subunit is likely to be dominated by 16S RNA. It is sensitive to kethoxal, RNase, or low concentrations of aurintricarboxylic acid (Pon & Gualerzi, 1976; Gualerzi & Pon, 1973; Pon et al., 1972; Sabol et al., 1973), independent of the presence or absence of many ribosomal proteins (Pon

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& Gualerzi, 1976; Laughrea et al., 1978a), and affected by the substitution of G791 by an A (Tapprich et al., 1989).

Materials and Methods

Buffers. Buffer A consisted of 10 mM magnesium acetate, 100 mM NH_4Cl, 0.5 mM EDTA, 1 mM dithiothreitol, and 20 mM Tris, pH 7.5. CMK was 20 mM magnesium acetate, 50 mM KCl, 1 mM dithiothreitol, and 50 mM sodium cacodylate, pH 7.2.

Chemicals and Enzymes. Escherichia coli MRE600, mid-log phase, was from Grain Processing Ltd., Muskateen, IA. AMV reverse transcriptase was from Pharmacia or Seikagaku. [α-^{35}S]dATP was from Amersham. MLV reverse transcriptase, ultrapure sucrose, and ultrapure urea were from Gibco. Phenol, Bistris, *N*-nitroso-*N*-ethylurea, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT) were from Sigma. Cacodylic acid and kethoxal monohydrate were from ICN. RNase V1, nuclease S1, and poly(U)–Sepharose 4B were from Pharmacia. DNase I was from Boehringer Mannheim. Dimethyl sulfate (DMS) was from Aldrich.

Ribosomes. 70S ribosomes, deprived of S1, were prepared from *E. coli* MRE600 as in Laughrea and Tam (1989). 30S subunits, deprived of S1, were derived from the 70S ribosomes as in Steitz et al. (1977) except that one cycle of sucrose gradient centrifugation was used ($200A_{260nm}$ units of ribosomes/SW28 tube) and that the NH_4Cl concentration was set at 750 mM in the concentration step, to ensure complete removal of residual S1 or initiation factors. The 30S subunits and the ribosomes were stored in buffer A in many small portions at −80 °C. Samples of 30S subunits were heat-activated by incubation at 42 °C for 25 min (Moazed et al., 1986) prior to chemical modification or nuclease hydrolysis.

Ribosomal Protein S1 and Initiation Factors IF1, IF2, and IF3. S1 was prepared by poly(U)–Sepharose and DEAE-cellulose chromatography (Laughrea & Tam, 1989). Pure initiation factors were obtained as described by Pawlik et al. (1981) and kindly provided by Dr. Claudio Gualerzi. The IF3 studied in this paper was the full-length form (Lammi et al., 1987). The activities of the factors and of S1 were checked as in Laughrea and Tam (1989). S1 and initiation factors were stored in many small samples at −80 °C.

Enzymatic Hydrolysis and Chemical Modification. Each assay contained 23 pmol of ribosomes or heat-activated 30S subunits incubated (10 min, 37 °C) with or without 69–460 pmol of S1, IF2, or IF3 in 40 μL of CMK or buffer A. In some experiments, 3–10 μM neomycin (Nm) was incubated for 30 min at 37 °C with 30S subunits either before or after the formation of 30S·IF3 complexes. Before treatment with an enzyme or a chemical reagent, the mixtures were left on ice for 10 min (Muralikrishna & Wickström, 1989). All chemical or enzymatic reactions were carried out at 37 °C in a final volume of 50 μL. For each enzymatically or chemically modified sample, there was a control sample deprived of the enzyme or the chemical but otherwise treated in the same way. Whenever cognate controls (e.g., 30S, 30S + S1, 30S + IF3) differed in the intensity of a given band, background was assumed to correspond to the intensity of the darkest band. Only if the chemical or the enzyme significantly increased the intensity of the darkest control counterpart was the position considered as having reacted.

RNase V1 digestion was carried out in CMK or buffer A with 0.04–0.4 unit (30S subunits samples) or 0.2–1.2 units (ribosome samples) of RNase V1. After incubation for 15 min, samples were deproteinized as in Muralikrishna and Wickström (1989) and finally stored at 0.77 pmol/μL in 1 mM EDTA (pH 7.0). Dimethyl sulfate (DMS) and carbodiimide (CMCT) modifications were carried out in CMK as in Muralikrishna and Wickström (1989). Kethoxal modifications were carried out in CMK (37 °C, 10 min, in the presence of 5.9 mg/mL kethoxal) as described by Stern et al. (1988b). Ethylnitrosourea modifications were carried out in buffer A as in Baudin et al. (1989), for 45 min (30S subunits) or 90 min (ribosomes).

We chose 37 °C for most of the probing experiments because ribosomes appear unable to initiate at temperature below 8 °C (Friedman et al., 1969). The various structure-specific probes used in this work, as well as their advantages and limitations, have been fully described (Ehresmann et al., 1987).

Primer Extension and Gel Analysis. The following oligodeoxynucleotide primers, named according to the position of the first transcribed nucleotide, were used as templates for reverse transcription: 683, 837, 906, 985, 1046, 1100, 1199, 1329, 1391, 1490, and 1508 (Moazed et al., 1986; Baudin et al., 1987). Hybridization to 16S RNA, primer extension, and gel electrophoresis were as in Moazed et al. (1986) except for the following changes. Extension reactions contained [α-^{35}S]dATP instead of [α-^{32}P]dATP, and proceeded at 37 or 55 °C (Lane et al., 1988), as indicated. AMV reverse transcriptase (Pharmacia) was the reverse transcriptase most often used, and was the one used to obtain the results presented in Figures 3 and 4. The reaction mixture was stopped by adding 0.5 volume of loading buffer (0.03% xylene cyanol and bromophenol blue in deionized formamide), heated at 90 °C for 2 min, and loaded (8 μL/sample) on a 38 × 50 cm SEQUIGEN (Bio-Rad) sequencing cell (2100 V × 2.5 h). After running, the gels were transferred to Whatman 3MM paper, dried, and autoradiographed for 1–4 days at −60 °C with Kodak XAR-5 film.

Results

Comment on the Methodology. Probing experiments were repeated several times on three entirely different preparations of S1, ribosomes, and 30S subunits (the IF3 always came from the same source). For each preparation, at least two independent modification experiments were generally done. All samples were probed with all primers. The exception is primer 683, which was used twice to probe RNase V1 and DMS reactivities, and never to probe kethoxal and CMCT activities. Only IF3 and S1 effects verified with all independent preparations are strongly emphasized here. Somewhat less reproducible results will also be mentioned: we would find them convincing if they were to be confirmed by an independent group.

Figure 1 shows that 86% of the bases located between G584 and U1506 were available for inspection, i.e., corresponded to blank control lanes. The actual number of bands in any control lane was actually about twice the number suggested by Figure 1, but half of the control bands could be removed by repeating the primer extension at a different temperature (e.g., 55 vs 37 °C) or with a reverse transcriptase from a different biological source. No one temperature or reverse transcriptase was ideal. Each condition would remove some control bands, but introduce others. Termination at the 3′ side of stems or at a U was 3 times more frequent than allowed by chance, while termination at an A was 6 times less frequent. In sum, 790 bases were effectively monitored within positions G584–U1506, and only a minority, if any, of the bands present in the control samples seems to come from RNA degradation.

Figure 2, summarizing a vast body of data from others, indicates that, in the region G584–U1506, about 200 bases

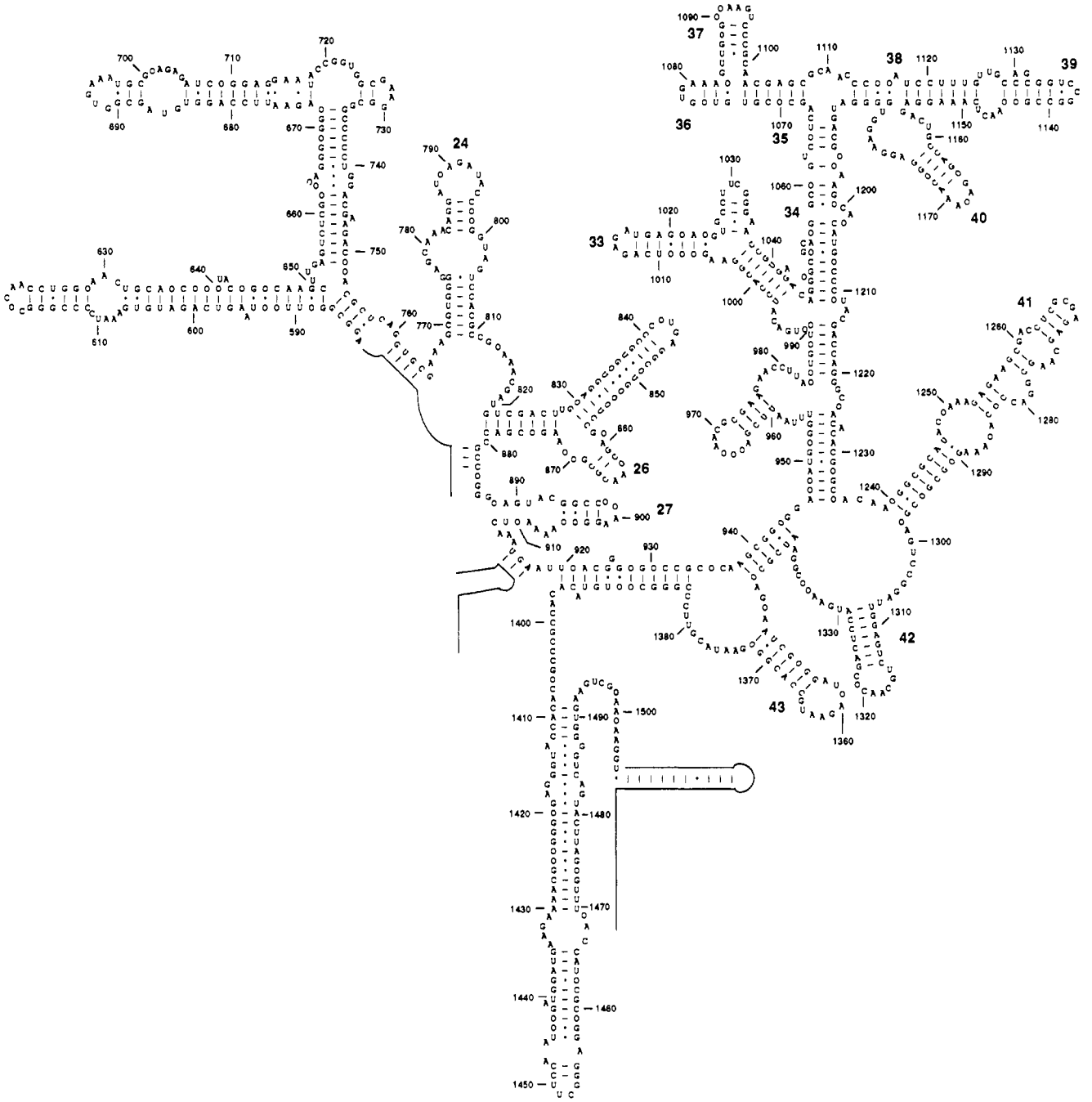

FIGURE 1: Schematic diagram of the secondary structure of *E. coli* 16S rRNA, with the 5′ domain mostly omitted. The secondary structure model is from Stern et al. (1988d); key helices (helices 34–40) are numbered according to Brimacombe et al. (1988) and have been incorporated into Noller's model by Raué et al. (1988). Canonical base pairs are represented by bars and noncanonical ones by dots. A, C, G, U, bases whose reactivity was probed by primer extension; O, bases whose reactivity could not be probed due to stop of reverse transcriptase; (–), bases outside the scope of this paper.

are chemically or enzymatically accessible in the 30S subunit and that the vast majority of hairpins, helices, and single-stranded stretches of 16S RNA have 1 or several exposed bases. This fact forces us to scan the whole 584–1506 region in our search for interaction sites between S1, IF3, and rRNA residues, hence our use of 11 different primers to permit unambiguous inspection of every residue of this region.

We found that independent gel electrophoretic analysis of the same material yielded identical results but that independent chemical modification or RNase digestion experiments, or independent preparations of ribosomes, could yield some variation in banding patterns. We decided to pay attention only to results which were clearly seen in ≥50% of five or more independent experiments and to discuss in detail only those obtained in >75% of five or more experiments.

Interaction of S1 and IF3 with the 30S Subunit or the Ribosome As Probed by RNase V1. RNase V1 cleaved the 30S subunits at 21 positions and the ribosomes at 15 positions (Table I). S1 reduced RNase V1 cleavage at G1164 by more than 60%, both with the ribosomes and with the subunits (Figure 3 and Table I). In early experiments, done on 30S subunits only, effects of S1 were seen at positions 797, 796, and 784, but these effects were not seen in any of the later experiments (Table I).

IF3 always strongly prevented cleavage at A1408, a site which is not available to RNase V1 in the ribosome (Vassilenko et al., 1981; Table I). This is illustrated in Figure 4A which also shows that 3 μM Nm affects neither the RNase V1 reactivity at A1408 nor the ability of IF3 to footprint there, even though Nm prevents DMS reaction at the A1408 of 30S

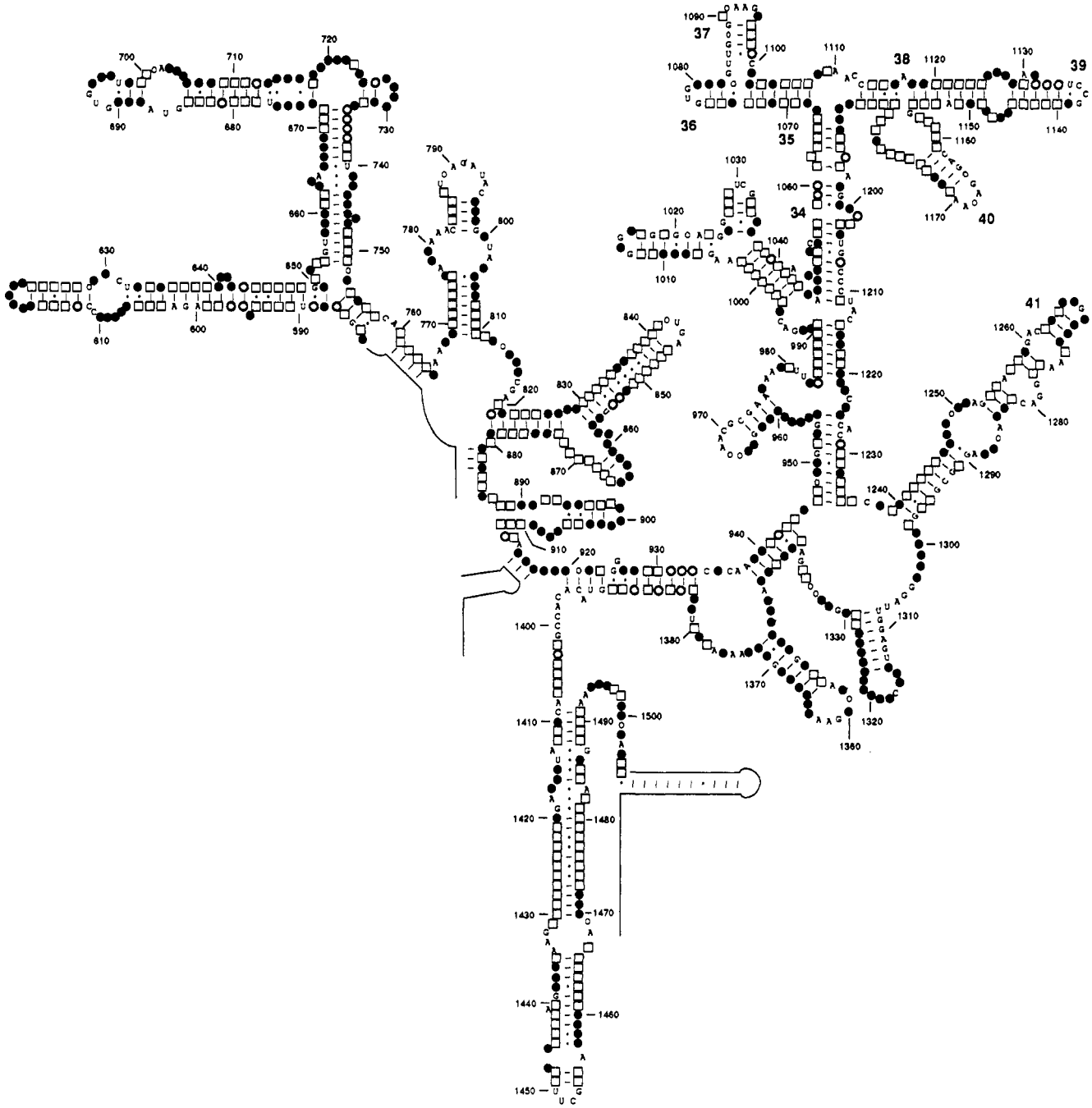

FIGURE 2: Schematic diagram of the secondary structure of *E. coli* 16S rRNA, emphasizing, in order (a) bases available to at least one structural probe in the 30S(–S1) subunit (A, C, G, U), (b) nucleotide residues available to at least one structural probe in naked 16S rRNA but fully protected by proteins S2–S21 (●), phosphate of the 30S subunit available to ethylnitrosourea (□), or nucleotide residues unprobed due to stop of the reverse transcriptase (o) or unreactive to any probe in naked 16S RNA (○). These data are integrated from Meier and Wagner (1985), Vassilenko et al. (1981), Moazed et al. (1986), Baudin et al. (1987, 1989), Mougel et al. (1987, 1988), Stern et al. (1988a,b), Powers et al. (1988a,b), Svensson et al. (1988), and Muralikrishna and Wickström (1989). When results were dual or dissimilar, evidence for availability took precedence over evidence for full protection; thus, this diagram tends to overemphasize base availability and to underestimate the number of fully protected residues. For clarity, the diameter of the stems and the length of the single-stranded stretches are reduced by about half relative to what would be reasonable given the base-pair spacing. RNase V1 cleavages claimed by Muralikrishna and Wickström at 722, 737, and 764 are not accepted (see Discussion).

subunits (Moazed & Noller, 1987; our unpublished results). These results apply even when 10 μM Nm is preincubated with 30S subunits before the addition of IF3 (data not shown). Finally, in 7 experiments (out of 11) where RNase V1 cleaved at A1500, IF3 reduced cleavage activity by more than 30%, 6 times out of 7 (Table I).

Interaction of S1 and IF3 with the 30S Subunit or the Ribosome As Probed by Dimethyl Sulfate, Kethoxal, and CMCT. Kethoxal and CMCT reacted at 46 different residues on 30S subunits or ribosomes (Table I). S1 and IF3 had no effect on these reactions.

DMS reacted at 37 different A's or C's. Effects of S1 or IF3 were observed, none, however, as striking as their influence on RNase V1 cleavage at 1164 and 1408 (Table I). S1 weakly protected A1503 and C1302 and stimulated the reactivity of A1004 and A901. A1503 strongly reacted with DMS both in 30S subunits (in six experiments out of seven) and in ribosomes (in five experiments out of five). In three experiments out of six, S1 weakly protected A1503 of 30S subunits. In none did S1 protect A1503 of ribosomes. C1302 did not react in ribosomes and reacted very weakly or not at all in 30S subunits. However, whenever it reacted, the reaction was

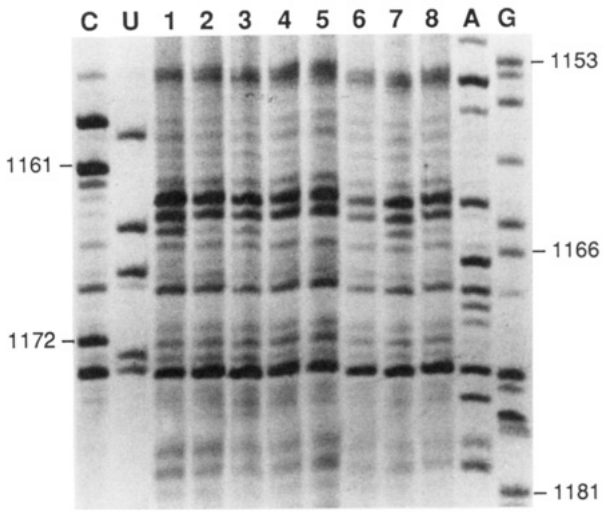

FIGURE 3: Typical autoradiogram showing the results of RNase V1 probing as monitored by primer extension at 37 °C using the 1199 primer. 0.6 unit of RNase V1 was used in samples 1–4, 0.2 unit in samples 7–8, and none in the control samples 4–6. Complexes probed: lanes 1, 4, and 7, 70S; lanes 2 and 5, 70S + 15-fold molar excess of S1; lanes 3, 6, and 8, 70S + 4-fold molar excess of S1. Lanes C, U, A, and G are sequencing products generated in the presence of ddGTP, ddATP, ddTTP, and ddCTP, respectively.

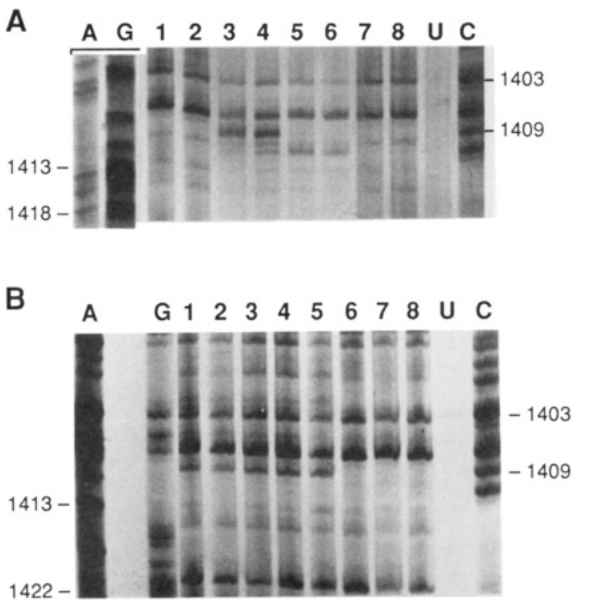

FIGURE 4: Typical autoradiograms showing the results of RNase V1 and DMS probing as monitored by primer extension at 55 °C using the 1490 primer. (A) RNase V1 probing. 0.4 unit of RNase V1 was used in samples 3–6, and none in the control samples 1, 2, 7, and 8. Complexes probed: lanes 1 and 3, 30S; lanes 2 and 5, 30S + 10-fold molar excess IF3; lanes 4 and 7, 30S in the presence of 3 μM Nm; lanes 6 and 8, 30S + 3 μM Nm + 10-fold molar excess IF3. (B) DMS probing. No DMS was present in the control samples 6–8. Complexes probed: lanes 1 and 6, 70S; lane 2, 70S + 15-fold molar excess S1; lanes 3 and 7, 30S; lane 4, 30S + 10-fold molar excess IF3; lanes 5 and 8, 30S + 15-fold molar excess IF3.

always reduced by S1. A1004 was consistently reactive in 30S subunits but not affected by S1 (Table I). With ribosomes, A1004 was reactive in four independent experiments out of six. In three of these four experiments, S1 increased somewhat its reactivity (Table I). No reaction was ever observed at A901 of ribosomes or 30S subunits. However, in four experiments out of seven, S1 made slightly reactive the A901 of ribosomes and 30S subunits (Table I).

Since IF3, but not Nm, prevents RNase V1 cleavage at A1408 (Figure 4A), it is noteworthy that IF3 does not prevent DMS reaction at A1408 (Figure 4B). Thus, we are not aware of any compound that can block both the DMS and the RNase V1 reactivity of A1408. It is also noteworthy that Nm does not bind normally to 30S·IF3 complexes. Ten micromolar Nm was incubated with 30S subunits or preformed 30S·IF3 complexes. The DMS reactivity of A1408 in 30S subunits was reduced by ~75% in the presence of Nm. That of the complex was left unchanged (data not shown). In brief, IF3 and IF3 + Nm prevent RNAse V1 access to A1408 but permit DMS reaction with it; Nm prevents DMS access to A1408 but permits RNAse V1 attack at A1408.

To be complete, we report results seen more than twice but in less than 50% of the experiments. They involve weak effects of S1 at A759, A782, A892, and A1408. We do not take these effects seriously, for lack of reproducibility, but we are not sure that they are artifacts. We see these residues as areas where a conditional effect of S1 has not been ruled out. In three experiments out of seven, S1 reduced the DMS reactivity of the A892 of 30S subunits (Table I). A892 and A901 belong to the same hairpin, not far from the region 861–888, which may have been cross-linked to S1 (Golinska et al., 1981; Brimacombe et al., 1988). However, S1 never protected A892 in ribosomes. The A1408, A782, and A759 of 30S subunits were always reactive to DMS. S1 protected A1408 in 3 experiments out of 7 and the 2 others in 3 experiments out of 10 (Table I). Only preliminary experiments were done with ethylnitrosourea. Weak reactivities were observed, and the subject was not investigated further.

Discussion

G1164, C1302, A1004, A901, A1503, and S1. The strongest effect of S1 was protection of hairpin 40 (residues 1161–1175 of the 3′ major domain) against RNase V1 attack. This increases the consistency of current knowledge on the structure of the 30S subunit. The assembly of S1 within the 30S subunit requires S9, with marginal contributions from S2, S3, and S10 (Laughrea & Moore, 1978b), and the nearest protein neighbors of S1 are S2, S19, and S9 (Capel et al., 1988). Proteins S2, S3, S9, S10, and S19 (and S7 and S14) are strongly associated with the 3′ major domain of 16S RNA, and only with that domain (Stern et al., 1988d; Wiener et al., 1988; Powers et al., 1988a,b; Chiaruttini et al., 1986; Hajnsdorf et al., 1986). Hairpin 40 was located not far from S2, S3, and S9 (Brimacombe et al., 1988; Stern et al., 1988d). Our data, together with those of Powers et al. (1988b), suggest that S1 and S2 have additive effects at G1164, the dominant role being played by S1. This has a happy consequence. With helices 35–40 (Figure 1) being constrained by cross-links with each other and with proteins S3, S9, and S10, it proved impossible for Stern et al. (1988d) to make S2 and hairpin 40 touch. At best, they could position hairpin 40 halfway between S2 and the cluster S3–S7–S9–S10, i.e., exactly where S1 is located in the neutron map of Capel et al. (1988). We are tempted to imagine direct effects of S1 at G1164 and allosteric effects of S2 at this position.

The protection of C1302 from DMS attack by S1 is also in harmony with previous knowledge. C1302 is at the base of hairpin 41 (Figure 1), which has been cross-linked to hairpin 39, near hairpin 40. C1302 is in the middle of many strong S7 footprints, as well as equidistant to several sites of strong S9 footprints (Brimacombe et al., 1988; Powers et al., 1988a). S7 has been cross-linked to A1238–C1240, opposite C1302 (Brimacombe et al., 1988). Thus, parts of S1 may be located near S7 and not far from S9, in accord with assembly mapping (Laughrea & Moore, 1978b). S1 increased somewhat the DMS reactivity of A1004 in 70S ribosomes. A1004 is in the middle of very strong S19 footprints (Powers et al., 1988a),

Table I: Effects of S1 and IF3 on Enzymatic and Chemical Probing of 16S RNA[a]

nucleotide position	RNase V1 30S(–S1)	RNase V1 70S(–S1)	RNase V1 30S(–S1) + S1	RNase V1 70S(–S1) + S1	RNase V1 30S(–S1) + IF3	nucleotide position	RNase V1 30S(–S1)	RNase V1 70S(–S1)	RNase V1 30S(–S1) + S1	RNase V1 70S(–S1) + S1	RNase V1 30S(–S1) + IF3
A1500	(±)	0			↓	G1020	++	++			
U1485	(+)	0				A1019	(+)	(+)			
A1408	+	0			↓↓	G874	±	±			
G1310	(+)	0				C840	++	++			
G1294	(±)	0				C839	++	++			
U1264	+	(±)				C797	+	±	*		
G1164	+	+	↓↓	↓↓		C796	+	±	*		
C1162	+	+				A784	+	+	*		
G1039	+	+				G773	++	±			
C1038	+	+				U772	(+)	0			
C1037	+	+									

nucleotide position	dimethyl sulfate 30S(–S1)	dimethyl sulfate 70S(–S1)	dimethyl sulfate 30S(–S1) + S1	dimethyl sulfate 70S(–S1) + S1	dimethyl sulfate 30S(–S1) + IF3	nucleotide position	dimethyl sulfate 30S(–S1)	dimethyl sulfate 70S(–S1)	dimethyl sulfate 30S(–S1) + S1	dimethyl sulfate 70S(–S1) + S1	dimethyl sulfate 30S(–S1) + IF3
A1503	++	+	(↓)			C1031	±	±			
A1483	±	±				A1014	±	±			
A1468	(±)	(±)				A1004	+	(+)		(↑)	
A1456	+	+				A969	±	±			
A1441	±	±				A968	±	±			
A1418	+	+				A901	0	0	(↑)	(↑)	
A1408	+	+	*			A892	+	±	*		
C1400	(±)	(±)				A845	(+)	(+)			
C1379	±	±				A831	(±)	(±)			
A1375	±	±				C817	(±)	(±)			
A1306	(+)	(±)				A816	(±)	(±)			
C1302	(±)	0	↓			A814	(±)	(±)			
A1275	(±)	(±)				C795	(+)	(±)			
A1261	++	++				A794	(+)	(±)			
A1256	±	±				A790	(+)	(±)			
C1192	++	+				A782	++	+	*		
A1188	(±)	(±)				A759	++	++	*		
C1137	±	(±)				A665	+	+			
A1110	±	(±)									

nucleotide position	kethoxal 30S(–S1)	kethoxal 70S(–S1)	nucleotide position	CMCT 30S(–S1)	CMCT 70S(–S1)	nucleotide position	kethoxal 30S(–S1)	kethoxal 70S(–S1)	nucleotide position	CMCT 30S(–S1)	CMCT 70S(–S1)
G1487	++	+	U1451	++	++	G1131	+	±			
G1453	++	++	U1381	+	+	G1108	(±)	(±)			
G1432	+	+	G1297	+	+	G1039	+	+			
G1361	+	not done	U1286	±	±	G1032	+	+			
G1338	+	not done	G1278	●	±	G1026	+	+			
G1300	+	+	U1212	+	+	G1015	+	+			
G1297	++	++	U1183	(±)	(±)	G1013	+	+			
G1278	++	++	G1039	±	±	G1003	±	±			
G1272	(±)	(±)	U1030	+	+	G973	±	±			
G1270	(±)	(±)	U973	±	±	G971	±	±			
G1260	(±)	(±)	U843	++	++	G926	+	+			
G1190	+	+	U842	++	++	G890	(±)	(±)			
G1182	±	±	U820	(+)	not done	G844	+	+			
G1178	(±)	(±)	G818	(+)	not done	G818	±	±			
G1166	+	+	U801	+	not done	G803	(±)	(±)			
G1160	(±)	(±)	U793	+	not done	G791	+	(±)			
G1156	±	±				G778	(±)	(±)			
G1138	+	±									

[a] Data summarize visual estimates of relative band intensities from 5–11 experiments. Reactivities are summarized qualitatively by the symbols, 0, ±, +, and ++ in order of ascending reactivity. The effects of the added proteins are summarized by the symbols ↓ and ↓↓ to indicate, respectively, that the reactivity has been reduced by 30–60% or ≥60% or by the symbol ↑ to indicate that the reactivity has been increased by 30–60%. A blank indicates that the reactivity is unchanged (±30%) by the added proteins. Data reported in parentheses have been seen in 50–75% of the experiments. All other data have been seen in >75% of the experiments. An asterisk means that an effect of S1 or IF3 has been seen more than twice but in less than 50% of the experiments. No effects of S1 or IF3 were observed with CMCT and kethoxal.

and S19 is the second nearest protein to S1 (Capel et al., 1988). Of course, A1004, C1302, and G1164 are all members of the 3′ major domain.

The head of the 30S subunit contains S2, S3, S7, S10, S14, and S19 and is devoid of sequences from the central and the 3′ minor domains (S9 and the 3′ major domain of 16S RNA have not yet been mapped by immunoelectron microscopy). These data, together with our results, strongly suggest that the 3′ major domain and a significant part of S1 are located (or have conformational effects) in the head of the 30S subunit.

Yet S1 is longer (possibly more than twice longer) than any interprotein distance in the head of the 30S subunit (Capel et al., 1988; Guinier, 1939). That S1 sticks out of the ribosome is supported by immunoelectron microscopy and NMR data (Walleczek et al., 1990; Cowgill et al., 1984), and is consistent with neutron-scattering experiments indicating that non-cross-linked S1 is located 69 ± 23 Å away from the center of scattering mass of the 30S subunit (Laughrea et al., 1978b).

Our finding that S1 influenced the accessibility of A901 (central domain) and A1503 suggests that part of S1 is located in (or affects the conformation of) the platform-cleft area (Oakes et al., 1990; Lasater et al., 1989). This is consistent with cross-linking and immunoelectron microscopy experiments. A901 is located in hairpin 27, which is protected to some extent by S11 and S12 (Stern et al., 1988c). S1 influences the reactivity of S12 (Michalski et al., 1978), and S1 and S11 are essential for restoration of the Phe-tRNA binding activity of tetranitromethane-inactivated ribosomes (Fanning et al., 1978).

We find the evidence for weak S1 protection at A1503 a little puzzling. Laughrea and Moore (1978a) had shown that S1 does not interact with region 1494–1542 of 30S subunits, unless the subunits are inactivated. In the inactive conformation, 30S subunits can bind two molecules of S1, and the second binding site depends on the sequence 1494–1542 for its existence (Laughrea & Moore, 1978a). We think that the protection of A1503, seen only with 30S subunits, reflects an allosteric effect of S1 or a mixture of allosteric effect and footprinting of S1 bound to its second binding site. Anyhow, among the S1 footprints at A901, A1004, G1164, C1302, and A1503, the last one, seen only in 50% of experiments with 30S subunits, was the least reproducible.

A1408, A1500, and IF3. Residues 1494–1542 (the colicin E3 fragment) are essential for IF3 binding to the 30S subunit under high-pressure conditions, but dispensable at lower pressure (Laughrea et al., 1978a), suggesting that IF3 binds close to some of these residues. This is supported by our finding that IF3 prevents RNase V1 cleavage at A1500 of the 30S subunit, by the cross-linking of IF3 to region 1506–1529 of the 30S subunit (Ehresmann et al., 1986), and by the fact that in 1 mM Mg^{2+} IF3 influences nuclease accessibility to residues A1499–U1506 of the isolated colicin E3 fragment (Wickström, 1983).

However, the dominant footprint of IF3 was found at A1408, located due to secondary structure alone, within one IF3 diameter from the colicin E3 fragment. Four types of biologicals leave a footprint at A1408: IF3 and the 50S subunit protect it from RNase V1 but not DMS attack, while A-site tRNA and antibiotics such as Nm, gentamicin (Gm), kanamycin (Km), or paromomycin (Pm) protect it from DMS attack (Vassilenko et al., 1981; Moazed & Noller, 1987, 1990; this paper). Since C1400 and G1401 are strongly implicated as P-site determinants, and A1492 and A1493 as A-site determinants (Moazed & Noller, 1990; Prince et al., 1982), we propose, given the hybrid or pleiotropic properties of C1404, C1409, G1491 (Denman et al., 1989; De Stasio & Dahlberg, 1990), and A1408, that A1408 is located at the interface (or imaginary border) separating A-site and P-site, and influences the function of both.

Does Nm prevent IF3 binding to the 30S subunit? Pon et al. (1972) found that 14 μM Nm reduced IF3 binding by 80% at 30 °C. Thibault et al. (1972) found that 100 μM Nm did not inhibit IF3 binding at 4 °C. In our hands, 10 μM Nm, when incubated for 30 min at 37 °C in the presence of 30S subunits, did not prevent IF3 from blocking RNase V1 attack on A1408. However, IF3 did prevent Nm from blocking DMS attack at A1408. The simplest interpretation of our results is that IF3 and Nm do not share the same binding site to an obvious extent but IF3 sterically hinders access of Nm to its own binding site. Because we have shown that IF3 does not prevent DMS attack at A1408, its binding site, and that of Nm, cannot include the whole A1408 residue, but may include part of it.

Comparison with Other Results. Muralikrishna and Wickström (1989) (M&W) have studied the influence of IF3 and S1 on residues 690–850. They have not seen any effect of S1, like us in this region of 16S RNA. However, they have reported IF3 footprints on U697, U701, G722, C737, C764, U793, A814, G833, G847, and G849, when we have seen none. Here is the best we can do to reconciliate our two sets of results. First, let us distinguish between the data of M&W obtained with RNase V1 (the effects of IF3 at U697, G722, C737, C764, G833, G847, and G849) and with other probes (at U701, U793, and A814). The RNase V1 data of M&W are dominated by the region 700–770. They show that RNase V1 does not cleave in the region 700–770; IF3 does not stimulate any RNase V1 cleavage in this region; the only prominent features are three strong control bands at G722, C737, and C764; neither IF3 nor RNase V1 has any effect on these control bands, but RNase V1 + IF3 make them disappear. Dr. E. Wickstrom agrees with these statements of facts (personal communication) which represent a correction of what was stated in M&W. We conclude that M&W have seen an effect of RNase V1 in the presence of IF3 rather than an effect of IF3 as assayed by RNase V1 cleavage analysis. The simplest interpretation is that such results are artifacts due to excess RNase V1 hits per 30S subunit. In agreement, M&W have reported several RNase V1 cleavage sites (e.g., at positions 697, 833, 846, 847, 848, and 849) heretofore unseen by at least four other groups (Vassilenko et al., 1981; Svensson et al., 1988; Mougel et al., 1988; Table I). Another interpretation is that, for unclear reasons, our 30S subunits have a tighter, less accessible structure than those of M&W or that the two IF3 preparations, though both full-length forms, somehow differ in structure. As for the chemical probing, we distinguish between weak and strong effects and allow that weak effects imply nonembarassing discrepancies with our data. Strong IF3 footprints were seen at U701 and A814. We cannot strongly comment on the first footprint due to a prominent control band in our gels at this position. We have observed DMS attack on A814 (Table I) and cannot explain why M&W saw a stimulatory effect of IF3 at this position while we did not. In sum, most of our data are compatible with those of M&W in the region 690–850. The only unexplained discrepancy is at the level of A814.

Neither our work nor that of M&W provides strong evidence for the idea (Tapprich et al., 1989; Santer et al., 1990) of a direct interaction between IF3 and the loop (787–795) of hairpin 24. We have found that A790, G791, U793, and A794 were reactive to one or another probe. None of these residues were protected by IF3, except for a minor discrepancy at U793: M&W found that it was very weakly protected. Together with the results of Tapprich et al. (1989) and Santer et al. (1990), our data suggest that hairpin 24 promotes IF3 binding through allosteric effects rather than direct interaction. This is supported by M&W since one of their strongest results is that IF3 increases the reactivity of A814 in hairpin 24.

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Crystal Structure of the Cytochrome P-450$_{CAM}$ Active Site Mutant Thr252Ala[†,‡]

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ABSTRACT: The crystal structure of a cytochrome P-450$_{CAM}$ site-directed mutant in which the active site Thr252 has been replaced with an Ala (Thr252Ala) has been refined to an *R* factor of 0.18 at 2.2 Å. According to sequence alignments (Nelson & Strobel, 1989), Thr252 is highly conserved among P-450 enzymes. The crystallographic structure of ferrous camphor- and carbon monoxide-bound P-450$_{CAM}$ (Raag & Poulos, 1989b) suggests that Thr252 is a key active site residue, forming part of the dioxygen-binding site. Mutation of the active site threonine to alanine produces an enzyme in which substrate hydroxylation is uncoupled from electron transfer. Specifically, hydrogen peroxide and "excess" water are produced instead of the product, 5-*exo*-hydroxycamphor. The X-ray structure has revealed that a local distortion in the distal helix between Gly248 and Thr252 becomes even more severe in the Thr252Ala mutant. Furthermore, a solvent molecule not present in the native enzyme is positioned in the dioxygen-binding region of the mutant enzyme active site. In this location, the solvent molecule could sterically interfere with and destabilize dioxygen binding. In addition, the active site solvent molecule is connected, via a network of hydrogen bonds, with an internal solvent channel which links distal helix residues to a buried Glu side chain. Thus, solvent protons appear to be much more accessible to dioxygen in the mutant than in the wild-type enzyme, a factor which may promote hydrogen peroxide and/or water production instead of substrate hydroxylation. On the basis of crystallographic and mutagenesis data, a proton delivery pathway involving residues Lys178/Arg186, Asp251, and Thr252 is proposed for wild-type P-450$_{CAM}$. Coordinates of structures discussed in this paper have been submitted to the Brookhaven Protein Data Bank (Bernstein et al., 1977).

The cytochrome P-450 superfamily of enzymes catalyzes many different types of oxidative reactions involved in endogenous metabolic processes including steroid hormone and bile acid biosynthesis and fatty acid metabolism. These enzymes are also involved in detoxification of foreign compounds and have been implicated in carcinogenic transformation (Nebert et al., 1981; Nebert & Gonzalez, 1987; Anders, 1985). Recent reports indicate that P-450s also may be involved in yeast spore wall formation (Eckerstorfer et al., 1991) and possibly in fungal denitrification pathways (Shoun, 1991). The enormous variety of processes P-450 enzymes are involved in as well as the seemingly limitless number of their potential substrates are well represented by a recent review (Guengerich, 1991). As a result of their broad involvement in biochemical processes, there is much interest in structure–function relationships of P-450s with aims including the design of P-450 inhibitors and the engineering of novel P-450s with specificities

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[‡] Coordinates have been deposited in the Brookhaven Protein Data Bank and assigned numbers as follows: 2CP4, camphor-bound Thr252Ala mutant P-450$_{CAM}$ (data set 252A1); 3CP4, 11-week adamantane-bound wild-type P-450$_{CAM}$ (data set ADAM8); 4CP4, recombinant wild-type camphor-bound P-450$_{CAM}$ (data set NCAM4).
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